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Human renal fibroblasts modulate proximal tubule cell growth and transport via the IGF-I axis

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Human renal fibroblasts modulate proximal tubule cell growth and transport via the IGF-I axis. To determine the paracrine interactions involved in the tubulointerstitial response to progressive renal disease, the role of insulin-like growth factor-I (IGF-I) and its binding proteins (IGFBPs) in *in vitro* interactions between human proximal tubule cells (PTC) and renal cortical fibroblasts (CF) were studied in primary cell culture. PTC growth and transport were increased in the presence of CF-conditioned media (CF-CM), as shown by increased thymidine incorporation, cellular protein content and sodium-hydrogen exchange (NHE) activity, to 185 ± 31% ($P < 0.01$), 150 ± 18% ($P < 0.05$) and 195 ± 27% ($P < 0.01$) of the control values, respectively. IGF-I was produced by cultured CF at a rate of 64.6 ± 7.5 ng/mg protein/day. Exogenous IGF-I applied to PTC provoked similar enhancement of growth and NHE activity as CF-CM and the stimulatory effect of CF-CM was blocked by specific immunoneutralization of IGF-I receptors. These receptors were threefold more abundant on PTC basolateral versus apical membranes. IGF binding proteins (IGFBP)-2 and IGFBP-3 were secreted by CF at rates of 694 ± 88 and 1769 ± 45 ng/mg/day, with the release of IGFBP-3 being enhanced in the presence of PTC-CM (120.0 ± 9.7% of control, $P < 0.01$). Moreover, the addition of CF-CM to PTC increased cell-associated IGFBP-3 on PTC surfaces, without changes in IGF-I receptor numbers or affinity and without changes in PTC mRNA for IGFBP-3. Des(1-3)IGF-I, an analog that binds to the IGF-I receptor but not to IGFBPs, provided a less potent stimulus for PTC growth compared with IGF-I, indicating that cell-associated IGFBP-3 facilitates the action of IGF-I on PTC. The results support important paracrine roles for both IGF-I and IGFBPs in the interstitial regulation of proximal tubule growth and transport.

The ontogeny, growth and function of many epithelial tissues is regulated by the adjacent stroma [1]. However, despite the fact that over 60% of the outer proximal tubular surface is in direct contact with the cortical interstitium [2], paracrine interactions between mesenchymal and epithelial tissues within the kidney have received little study to date. Montessano, Schaller and Orci [3] have provided evidence that renal tubular cell growth and differentiation are under the influence of factors produced by fibroblasts by demonstrating that Madin-Darby canine kidney (MDCK) cells undergo mitogenesis and tubulogenesis in the presence of lung and skin fibroblasts, even when the cell populations are not in direct contact. Since both lung and skin fibroblasts are known to produce insulin-like growth factor-I (IGF-I) [4], a peptide that exerts diverse effects on proximal tubule cell (PTC) metabolism [5], growth [6] and transport [7], it is possible that renal cortical fibroblasts (CF) may also modulate adjacent PTC growth and function via the production of this cytokine.

Changes in intrarenal IGF-I have been shown to be important in several adaptive and pathologic renal conditions, including compensatory proximal tubular hypertrophy following nephron loss, diabetic nephropathy and tubular epithelial repair following toxic or ischemic insults [8–11]. More recently, it has been appreciated that the functions of IGF-I within the kidney are modulated by locally produced IGF binding proteins (IGFBPs) [11]. The precise cells within the kidney responsible for synthesis of the various components of the intra-renal IGF-I/IGFBP axis are not fully defined [11]. However, renal interstitial fibroblasts may well play a significant role in this axis as extrarenal fibroblasts are known to produce both IGF-I and a variety of IGFBPs [4, 12, 13]. Hence, the aim of the present *in vitro* study was to examine the paracrine IGF-I/IGFBP interactions between human CF and PTC populations and their influence on PTC growth and transport.

METHODS

Patients

Segments of macroscopically and histologically normal renal cortex were obtained aseptically from adult human kidneys removed surgically because of small (< 6 cm) renal adenocarcinomas ($N = 7$), pelviureteric transitional cell carcinoma ($N = 4$) or benign angiomyolipoma ($N = 1$). The average patient age was 66.7 ± 3.0 years and the male:female ratio was 7:5. Patients were otherwise healthy and were on no medications. Informed consent was obtained prior to each operative procedure, and the use of human renal tissue for primary culture was reviewed and approved by the Royal North Shore Hospital Human Research Ethics Committee.

Cell culture

The method for primary culture of human PTC is described in detail elsewhere [14]. Primary cultures of renal cortical fibroblasts were obtained by minor modifications of this method. Briefly, renal cortical tissue was dissected from the medulla, minced,

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digested with collagenase (class 2, 383 U/mg; Worthington, Freehold, NJ, USA) and passed through a 100 μm mesh. Filtered tissue was resuspended in 45% Percoll (Pharmacia, Uppsala, Sweden) and separated into four distinct bands by isopyknic ultracentrifugation. The lowermost band was removed for PTC culture. The uppermost band was removed for renal cortical fibroblast culture.

PTC were resuspended in serum-free, antibiotic-free, hormonally-defined media, consisting of 1:1 (vol/vol) Dulbecco's modified Eagle's media and Ham's F-12 (DMEM/F-12) (ICN Pharmaceuticals Inc., Costa Mesa, CA, USA), supplemented with 5 μg/ml human transferrin (Sigma Chemical Co., St. Louis, MO, USA), 5 μg/ml (0.87 μM) bovine insulin (Sigma), 0.05 μM hydrocortisone (Sigma), 10 ng/ml (1.64 nM) epidermal growth factor (Collaborative Research Inc., Bedford, MA, USA), 50 μM prostaglandin E_1 (Sigma), 50 nM selenium (Sigma) and 5 pM tri-iodothyronine (Sigma). The tubular fragments were plated at a density of 1.5 mg pellet/cm^2 (approximately 5000 to 7000 fragments/cm^2) in 75 cm^2 flasks (Corning, New York, NY, USA). Media were changed every 48 hours. The cells were incubated in humidified 95% air/5% CO_2 at 37°C and were subcultured at near-confluence using seeding densities of 4000 cells/cm^2. Such cells were designated passage 1. Cytologic examination of PTC preparations from all donors failed to reveal any evidence of cellular atypia. The ultrastructural, growth and transport characteristics of these cells and their responses to hormonal and cytokine stimulation have been previously studied in this laboratory and found to reproducibly exhibit the features of PTC *in vivo* [14].

Renal cortical fibroblasts (CF) were resuspended in antibiotic-free DMEM/F-12 containing 10% fetal calf serum (Trace Biosciences, Sydney, Australia) and were seeded at an initial density of 2.25 mg pellet/cm^2. Subculture at confluence was subsequently performed using a seeding density of 7000 cells/cm^2. Because doubling times were greater for CF than for PTC, the higher seeding densities used in the former group allowed the synchronization of the attainment of confluence in both populations at approximately five days after plating.

Cortical fibroblast characterization

The cellular viabilities of CF cultures were confirmed by Trypan blue exclusion [7]. Morphologic examination revealed pure populations of mitotic fibroblasts characterized by typical spindle or epithelioid morphologies (Fig. 1). Although PTC contamination accounted for up to 15% of cells in confluent primary cultures, fibroblasts rapidly overgrew PTC in the presence of 10% fetal calf serum, such that the numbers of PTC in subsequent passages were negligible. This was confirmed by uniform positive immunohistochemical staining for vimentin, an intermediate filament marker of mesenchyme [15], and negative staining for cytokeratin, an epithelial cell marker [15]. Mesangial cell contamination was excluded by negative staining for desmin (a marker of glomerular mesangial cells both *in vitro* and *in vivo*) [15]. Alpha-smooth muscle actin, a marker of pathologically activated fibroblasts (myofibroblasts) [16], could not be demonstrated in CF by either electron microscopy or immunohistochemistry. Confirmation of the renal cortical origin of fibroblasts was based on the known differential localization of 5′-ectocytonucleotidase (5NT) to cortical, but not medullary, fibroblasts [17]. Cells from the first four passages uniformly stained positive for 5NT, as determined by the lead precipitation technique of Wachstein and Meisel [18].

Experimental protocol

The role of IGF-I in paracrine CF-PTC interactions was evaluated by demonstrating that: (*a*) CF produce IGF-I and IGFBPs, (*b*) PTC express specific receptors for IGF-I, (*c*) IGF-I action on PTC is modulated by cell-associated IGFBPs, and (*d*) PTC secrete soluble factors into conditioned media which in turn regulate the production of IGF-I and/or IGFBPs by CF. Further experiments were designed to determine whether exposure of PTC to CF-derived IGF-I in either conditioned media or coculture experiments modulated PTC growth and sodium-hydrogen exchange (NHE) activity and whether these effects could be reproduced by exogenous IGF-I and blocked by a specific, neutralizing anti-IGF-I-receptor antibody. The effect of native IGFBPs on IGF-I-stimulated PTC growth was evaluated by comparing the relative actions of exogenous IGF-I and an IGF-I analog with markedly reduced affinity for IGFBPs.

All experiments were performed on quiescent, confluent, passage 2 PTC and CF. Cells were made quiescent by two washes followed by incubation for 24 hours in basic media (DMEM/F-12 containing 5 μg/ml human transferrin). PTC- or CF-conditioned media were obtained by incubating quiescent PTC or CF for 24 hours in 8 ml of fresh basic media in 25 cm^2 flasks. The media were then collected and centrifuged to remove cellular material.

Measurement of insulin-like growth factor binding protein production by cortical fibroblasts

CF-conditioned media (CF-CM) were initially screened for the presence of IGFBPs by ligand blot analysis, as described previously [13]. Ligand blots revealed a major band of relative molecular weight (M_r) = 42000 and an additional band of M_r = 32000. These bands were identified as IGFBP-3 and IGFBP-2, respectively, on the basis of M_r and immunoblotting with specific antisera (not shown). Quantitative measurement of IGFBP-3 and IGFBP-2 production was performed by assaying media conditioned for 4, 24 and 48 hours by CF in specific IGFBP-3 and IGFBP-2 radioimmunoassays (RIAs). The methods for these RIAs are described in detail elsewhere [19, 20].

Measurement of insulin-like growth factor-I production by cortical fibroblasts

IGFBPs were dissociated from IGF-I and were eliminated from CF-CM samples using the Bio-Spin P-10 separation method described by Mohan and Baylink [21]. IGF-I concentrations were then measured by assaying 50 μl in duplicate in a previously described RIA [22]. The absence of interfering IGFBPs from the fractions pooled for IGF-I measurements was confirmed by ligand blot analysis and IGF-I recovery rates exceeded 96%. Maximum specific binding of IGF-I tracer to antibody exceeded 30%. The detection limits of the assay were 0.1 to 50 ng/ml. Cross reactivity of IGF-II in this assay was negligible. The intra- and interassay coefficients of variation (cv) were less than 5% and 10%, respectively.

Detection of IGF-I receptors on proximal tubule cells

[^{125}I]-IGF-I binding. Using the chloramine-T method, 5 μg of recombinant human IGF-I (Kabi Peptide Hormones, Stockholm, Sweden) was iodinated with 1 mCi ^{125}I. The specific activity of [^{125}I]-IGF-I prepared in this fashion was approximately 200 μCi/μg. In order to independently evaluate the presence of IGF-I

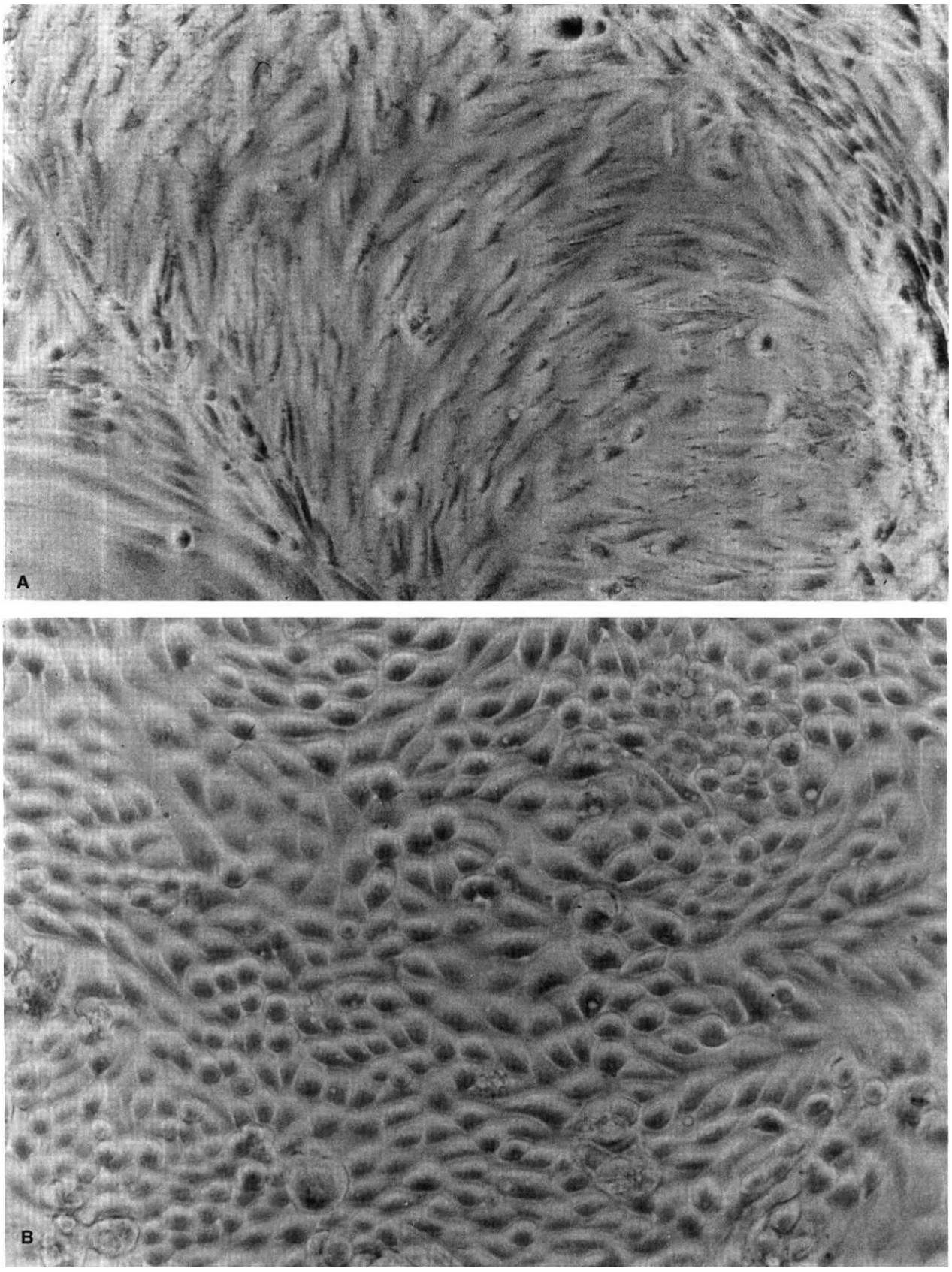

Fig. 1. (*A*) Typical confluent monolayer of human cortical fibroblasts (×500 magnification). The cells display typical plump, fusiform or stellate morphologies with smooth overlying surfaces and thin, sometimes ramified processes that contact adjacent cells via nexi. (*B*) Typical confluent monolayer of human proximal tubule cells (×500 magnification), displaying the characteristic "cobblestone" appearance.

binding sites on apical and basolateral membranes, PTC were grown on membrane filter inserts. After three washes with ice-cold PBS, the apical or basolateral aspects of PTC monolayers were respectively incubated in 200 μl or 300 μl basic media, containing [^{125}I]-IGF-I (200,000 cpm) in the presence or absence of various concentrations of unlabeled IGF-I (10^{-12} M to 10^{-6} M). Basic media was added to the contralateral compartments. All incubations were at 4°C for two hours. Media were then collected and further radioligand binding to PTC monolayers was stopped by three washes with ice-cold PBS. Cells were solubilized in 200 μl 0.2 M NaOH and were subsequently neutralized with an equivalent volume of 0.2 M HCl. Aliquots were submitted for protein determinations (Bio-Rad protein assay kit II; Bio-Rad, Hercules, CA, USA) and gamma counting. Nonspecific binding, defined as the amount of bound [^{125}I]-IGF-I in the presence of an excess of unlabeled IGF-I (1 μM), was subtracted from total binding to determine specific IGF-I binding. [^{125}I]-IGF-I degradation, determined by precipitation of 50 μl media with an equivalent volume of 20% trichloroacetic acid (TCA), was uniformly less than 10% under the above incubation conditions. Transepithelial leakage of [^{125}I]-IGF-I was also measured during each study and was consistently less than 2%.

Affinity cross-linking. Proximal tubule cells (PTC) were washed three times with cold PBS and then incubated with [^{125}I]-IGF-I (10^6 cpm) for two hours at 4°C in the presence or absence of 10^{-6} M unlabeled IGF-I, 10^{-6} M insulin or 5 μg/ml mouse monoclonal anti-IGF-I receptor antibody (αIR; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After a further three washes with PBS, IGF-I was covalently cross-linked to its receptor by exposing PTC for 15 minutes at 4°C to 500 μl basic media containing 0.25 mM disuccinimidyl suberate (Pierce Biochemicals, Rockford, IL, USA). The reaction was stopped by the addition of 1 ml 1 mM ethylenediaminetetra-acetic acid (EDTA)/10 mM Tris-HCl, pH 7.5, and the wells washed a further three times with PBS. Monolayers were solubilized in 100 μl fourfold concentrated Laemmli buffer (0.04 M Tris, 4% sodium dodecyl sulphate, 40% glycerol, 0.01% bromophenol blue, 100 mM dithiothreitol, pH 6.8) and separated by 7.5% SDS-PAGE under reducing conditions. Prestained molecular weight standards (Kaleidoscope, Bio-Rad) were processed in parallel. Gels were stained for three hours at 37°C with 0.1% Coomassie Brilliant Blue R-250 (Bio-Rad), 25% isopropanol and 10% acetic acid. After destaining for two hours at 37°C with 25% methanol, 10% acetic acid and 3% glycerol, the gels were dried and exposed to Hyperfilm-MP (Amersham) for 14 days at −80°C. Autoradiographic bands were quantitated by densitometry using the Kodak electrophoresis documentation and analysis system (Kodak Scientific Imaging Systems, Rochester, NY, USA).

To determine whether CF modulate IGF-I binding sites on PTC, the latter were also preincubated with CF-CM for 24 hours prior to radioligand binding and affinity labeling studies.

Proximal tubule cell feedback on cortical fibroblast production of IGF-I and IGFBPs

To determine whether PTC exerted feedback effects on the secretion of IGF-I and/or IGFBPs by CF, PTC-conditioned media (PTC-CM) or basic media were placed directly onto the CF monolayers. After 24 hours, the media were collected, centrifuged to remove any cellular material and assayed as described above.

Assessment of the growth effects of cortical fibroblasts on proximal tubule cells

Conditioned media experiments. Cellular protein content, an index of cell hypertrophy [23], and tritiated thymidine incorporation, an index of DNA synthesis and hence hyperplasia [23], were measured in PTC exposed for 24 hours to CF-CM, using previously outlined methods [7]. PTC incubated in basic media served as controls. The size of CF-derived soluble growth factors was inferred by evaluating the growth response of PTC to dialyzed CF-CM. CF-CM were initially dialyzed against fresh basic media using a 6 kDa cut-off dialysis membrane (Selby Anax, Sydney, Australia) prior to incubation with PTC. PTC were also exposed to PTC-CM to ensure that the effects of CF-CM were cell-type specific.

Coculture experiments. Coculture experiments were performed to determine whether any growth effects exerted by CF-derived soluble factors on PTC were subject to feedback regulation and to assess the potentially different effects of sustained low level release of growth factors by coincubated cells. Confluent monolayers of PTC grown on porous membrane filter inserts (Millicell-CM, 0.4 μm, 12 mm diameter; Millipore, Bedford, MA, USA) were placed over confluent CF monolayers grown in 24-well culture plates (Nunc, Roskilde, Denmark). Both cell populations were incubated together for 24 hours in basic media. Proximal tubule cells grown on inserts in 24-well plates without fibroblasts served as controls. Cellular protein content and thymidine incorporation were subsequently measured.

Assessment of the effects of cortical fibroblasts on proximal tubule cell sodium-hydrogen exchange activity

Sodium-hydrogen exchange (NHE) activities were measured in PTC monolayers incubated in basic media or CF-CM for 8, 16 or 24 hours. Intracellular pH (pH_i) recovery rates and H^+ efflux rates from cells following isosmotic, 20 mM NH_4Cl prepulses were measured in the presence or absence of 10 μM ethylisopropylamiloride (EIPA) by 2′,7′-bis(2 carboxyethyl)-5 [6]-carboxyfluorescein (BCECF) microspectrofluorimetry, as described previously [7]. NHE activity was defined as the component of H^+ efflux that was Na^+-dependent and inhibited by EIPA.

Inhibition of cortical fibroblast conditioned medium effects on PTC growth and NHE activity by IGF-I receptor immunoneutralization

To determine whether the effects of CF-CM on PTC growth and NHE activity could be inhibited by blocking the action of IGF-I at the IGF-I receptor, PTC were incubated in basic media or CF-CM, in the presence or absence of 5 μg/ml αIR, for 24 hours. Growth parameters and NHE activities were then measured as described above. A total of 5 μg/ml αIR was initially confirmed in pilot studies to abolish the growth effects of 50 ng/ml IGF-I on PTC (data not shown).

Assessment of the effects of exogenous IGF-I and des(1-3)IGF-I on proximal tubule cell growth

The role of PTC-associated IGFBPs in regulating the action of IGF-I on PTC was examined by comparing the concentration-response effects of exogenous IGF-I and des(1-3)IGF-I (Genentech, San Francisco, CA, USA) on PTC thymidine incorporation. Des(1-3)IGF-I is an IGF-I analogue that binds to the IGF-I

receptor with a similar affinity compared to IGF-I, but has markedly reduced binding to IGFBPs [8]. Thus, any difference in the relative actions of IGF-I and des(1-3)IGF-I reflects the modulatory effects of local IGFBPs.

IGFBP-3 mRNA analysis

Confluent, quiescent CF were incubated in PTC-CM or basic media for 24 hours. Total RNA was extracted from cell cultures by the guanidinium thiocyanate method [24] and quantified by spectrophotometric readings at $Absorbance_{260}$. For Northern blot analysis, RNA was denatured, diluted in sample buffer (50% deionized formamide, 6.6% formaldehyde, 1 × MOPS and 5.1 μg/ml ethidium bromide) and fractionated by electrophoresis through a 1.4% agarose gel (20 μg per lane). To confirm that similar amounts of RNA were loaded and to assess the integrity of RNA, ribosomal RNAs (28S and 18S) were visualized with UV transillumination. RNA was then transferred by capillary blotting to Hybond N membranes (Amersham) and fixed by baking at 80°C for two hours. Membranes were prehybridized at 42°C for two hours with hybidization buffer (50% deionized formamide, 5 × Denhardt's solution, 5 × SSPE, 7% SDS and 100 μg/ml denatured salmon sperm DNA) and were then hybridized at 42°C overnight with fresh buffer containing 30 × 10^6 cpm of a ^{32}P-labeled, human IGFBP-3 cDNA probe (kindly provided by Dr. S. Shimasaki, Scripps Institute, La Jolla, CA, USA). The blots were washed in 1 × SSC, 0.1% SDS at 42°C for 15 minutes and in 0.1 × SSC, 1% SDS at 42°C for 15 minutes. After washing, the membranes were exposed to Hyperfilm-MP (Amersham) between two intensifying screens at −80°C. The nylon membrane was then stripped by boiling in 0.1 × SSC solution containing 0.25% SDS for 60 minutes and then rehybridized with an 18S cDNA probe (a kind gift from D. Denhardt, New Jersey, USA). Autoradiographic bands were quantitated by densitometry. IGFBP-3 mRNA density was corrected for the densitometric intensity of 18S for each sample. For the purposes of comparison, this ratio was set at unity for control samples and other lanes on the same gel were expressed as a fold increase over this value.

Similarly, Northern blot analysis of IGFBP-3 mRNA levels was performed in PTC exposed to CF-CM or basic media for 24 hours, in order to determine the contribution of PTC IGFBP-3 production to PTC surface-bound IGFBP-3.

Statistical analysis

All studies were performed in triplicate from PTC cultures obtained from 4 to 10 separate human donors. Normal distribution of all data were confirmed by the Kolmogorov-Smirnov test. Results are expressed as mean ± SEM or as median (interquartile range), depending on the distribution of data. Statistical differences between groups were assessed by Student's *t*-test (or Mann-Whitney *U*-test for non-parametric analyses) or by one-way analysis of variance (ANOVA), depending on the number of comparisons being made. Pairwise multiple comparisons were made by Fisher's protected least significant differences test. IGF-I binding data were analyzed by Scatchard analysis [25]. Analyses were performed using the software package, Statview version 4.5 (Abacus Concepts Inc., Berkeley, CA, USA). *P* values less than 0.05 were considered significant.

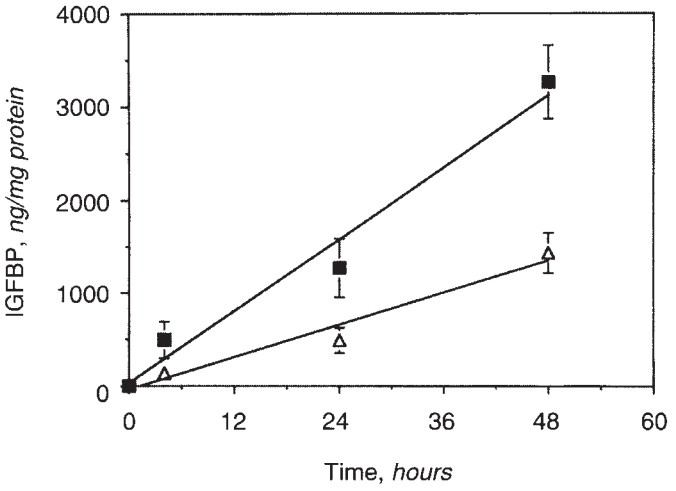

Fig. 2. Time courses for the release of IGFBP-2 (△) and IGFBP-3 (■) by cortical fibroblasts (CF) into culture media. Confluent, quiescent CF were incubated for the indicated time periods in basic media, which were then collected and assayed by specific IGFBP-2 and IGFBP-3 RIAs. Simple regression analyses yielded straight lines for IGFBP-2 ($y = 28.9x - 36.0$, $r^2 = 0.969$, $P < 0.0001$) and IGFBP-3 ($y = 64.9x + 31.6$, $r^2 = 0.999$, $P < 0.0001$).

RESULTS

Cortical fibroblasts produce IGF-I, IGFBP-2 and IGFBP-3

IGFBP-3 and IGFBP-2 were secreted into media by CF in a linear fashion during the first 48 hours (Fig. 2). CF-CM contained 3.9 ± 0.5 ng/ml IGF-I ($N = 18$) at 24 hours, which translated into a secretion rate of 64.6 ± 7.5 ng/mg protein/day or 42.6 ± 5.0 ng/10^6 cells/day.

In contrast, IGF-I and IGFBPs were not detectable in PTC-CM.

Proximal tubule cells predominantly express basolateral IGF-I receptors and exhibit cell-associated IGFBP-3

Scatchard transformation of the equilibrium radiolabeled IGF-I binding data for both apical and basolateral membranes revealed considerable asymmetry of IGF-I binding with the maximum specific binding capacities for apical membranes (53 ± 2 fmol/mg protein or 18,000 ± 1,000 binding sites/cell) being several-fold lower than for basolateral membranes (171 ± 6 fmol/mg protein or 56,000 ± 2,000 binding sites/cell; Fig. 3). The dissociation constants were similar for the two regions (1.26 nM vs. 1.31 nM, respectively). Over the two-hour time period, 6.2 ± 3.0% and 5.3 ± 1.9% of apically and basolaterally added [^{125}I]-IGF-I were degraded (P = not significant).

Affinity cross-linking experiments confirmed that human PTC expressed an [^{125}I]-IGF-I binding component with M_r 135,000 Da, consistent with the IGF-I receptor alpha-subunit (Fig. 4). Binding at this site was completely blocked by 10^{-6} M unlabeled IGF-I and by 5 μg/ml αIR. A second band (M_r 50,000) was identified as cell-associated IGFBP-3 on the basis of immunoblotting with a previously described, specific rabbit polyclonal IGFBP-3 antibody directed against human IGFBP-3 [19] (results not shown). Radioligand binding at this site was inhibited by 10^{-6} M unlabeled IGF-I, but not by αIR. Insulin, which exhibits less than 1% cross-reactivity with the IGF-I receptor and does not bind to

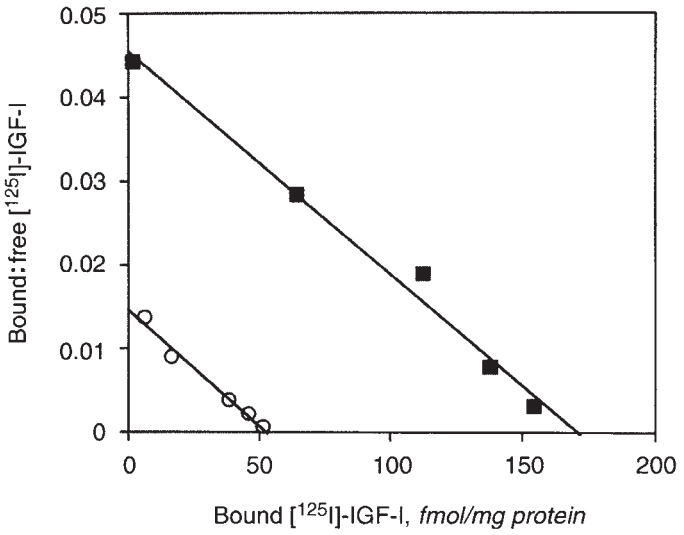

Fig. 3. Scatchard plots of [^{125}I]-IGF-I binding to the apical (○) and basolateral (■) membranes of human proximal tubule cells (PTC). PTC monolayers grown on porous membrane inserts were incubated for two hours at 4°C in media containing [^{125}I]-IGF-I, in the presence or absence of various concentrations of unlabeled IGF-I. Scatchard transformation yielded straight lines (basolateral, $y = -2.649 \times 10^{-4}x + 0.046$, $r^2 = 0.99$, $P < 0.01$; apical, $y = -2.737 \times 10^{-4}x + 0.015$, $r^2 = 0.98$, $P < 0.01$).

IGFBPs, resulted in partial inhibition of [^{125}I]-IGF-I binding to the M_r 135,000 band at a concentration of 10^{-6} M, but did not alter binding at the M_r 50,000 site. Following preincubation of PTC with CF-CM for 24 hours, the intensity of the M_r 50,000 band was increased by a median value of 3.9-fold (interquartile range 1.3 to 10.7, $N = 6$, $P < 0.05$), whereas that of the M_r 135,000 site was unchanged (median 1.0-fold, interquartile range 0.9 to 1.1, $N = 6$, P = not significant).

Proximal tubule cells exert feedback effects on cortical fibroblast release of IGFBP-3

Cortical fibroblasts exposed to PTC-CM for 24 hours secreted comparable amounts of IGF-I compared with controls (3.9 ± 0.5 vs. 3.9 ± 0.4 ng/ml, $N = 18$, P = NS). IGFBP-2 concentrations were also not significantly different between the two groups (6.3 ± 2.0 vs. 5.9 ± 1.9 ng/ml, respectively, $N = 30$). However, IGFBP-3 levels were increased in CF incubated in PTC-CM relative to controls (124.6 ± 9.0 vs. 91.2 ± 6.8 ng/ml, respectively, $N = 30$, $P < 0.01$). Measured IGFBP-3 secretion rates were 1162 ± 94 and 969 ± 59 ng/mg protein/day, respectively ($P < 0.05$).

Cortical fibroblasts stimulate proximal tubule cell growth

Proximal tubule cells cocultured with CF ($N = 24$) or exposed to CF-CM ($N = 30$) exhibited both increased cellular protein content and increased thymidine incorporation (Fig. 5). Dialysis of CF-CM prior to incubation with PTC ($N = 30$) did not modify the growth-promoting effects of CF-CM on PTC. Conversely, PTC incubated for 24 hours in PTC-CM ($N = 24$) showed no appreciable difference in growth parameters compared with controls.

Cortical fibroblasts stimulate proximal tubule cell sodium-hydrogen exchange activity

The resting pH_i of PTC exposed to CF-CM for 24 hours was significantly more alkaline than that of controls (7.14 ± 0.03 vs. 6.96 ± 0.03, respectively, $N = 54$, $P < 0.0001$). Following acid loading, the EIPA-sensitive pH_i recovery and H^+ efflux rates were also increased in CF-stimulated PTC (0.064 ± 0.007 U/min and 10.45 ± 1.42 mM/min, respectively) compared with control PTC (0.036 ± 0.006 U/min, $P < 0.05$ and 5.35 ± 0.91 mM/min, $P < 0.01$, respectively). Time course studies indicated that the onset of stimulation of PTC NHE activity by CF-CM was between 8 and 16 hours (Fig. 6).

IGF-I receptor immunoneutralization inhibits the effects of CF-CM on PTC growth and NHE activity

Although basic media containing αIR antibody had no intrinsic effect on PTC growth or NHE activity, the antibody completely blocked the stimulatory effects of CF-CM on PTC EIPA-sensitive H^+ efflux (Fig. 6), protein content (Fig. 7) and thymidine incorporation (Fig. 7). This confirmed that augmentation of both PTC growth and transport by CF was mediated via IGF-I.

Proximal tubule cell-associated IGFBP-3 facilitates the effects of IGF-I on PTC growth

Des(1-3)IGF-I, an IGF-I analog with markedly reduced binding affinity for IGFBPs, was significantly less potent than IGF-I with respect to augmentation of PTC thymidine incorporation (Fig. 8). The maximum stimulation of DNA synthesis by the two peptides was comparable (137 ± 20% vs. 140 ± 12% of control, respectively).

IGFBP-3 mRNA expression is unaltered in cortical fibroblasts by PTC-CM

Human CF and PTC both expressed significant amounts of IGFBP-3 mRNA (Fig. 9). Incubation of CF in PTC-CM for 24 hours, which stimulated CF secretion of immunoreactive IGFBP-3, was associated with a nonsignificant increase in the IGFBP-3 mRNA/18S RNA ratio compared with controls (median 1.2-fold, interquartile range 1.0 to 1.6, $N = 8$, $P = 0.07$). Conversely, PTC incubated in CF-CM for 24 hours also did not significantly alter their IGFBP-3 mRNA expression compared with controls (median 0.9, interquartile range 0.5 to 1.1), despite the significant increase in PTC-associated IGFBP-3 demonstrated by affinity labeling studies.

DISCUSSION

As histological changes in the tubulointerstitial environment are a major determinant of renal disease progression, even where there is primary glomerular injury [26], the pathogenesis of the observed heterogeneous areas of tubular atrophy and hypertrophy is the subject of current investigation in a number of laboratories. The present study demonstrates reciprocal paracrine regulation of human proximal tubule cell (PTC) growth and transport by renal cortical fibroblasts (CF) *in vitro*, which is mediated by the IGF-I/IGFBP axis. Specifically, we have demonstrated that CF secrete IGF-I, IGFBP-2 and IGFBP-3, and that the secretion of IGFBP-3 is subject to feedback regulation by soluble factors released by PTC. PTC, which have an abundance of IGF-I binding

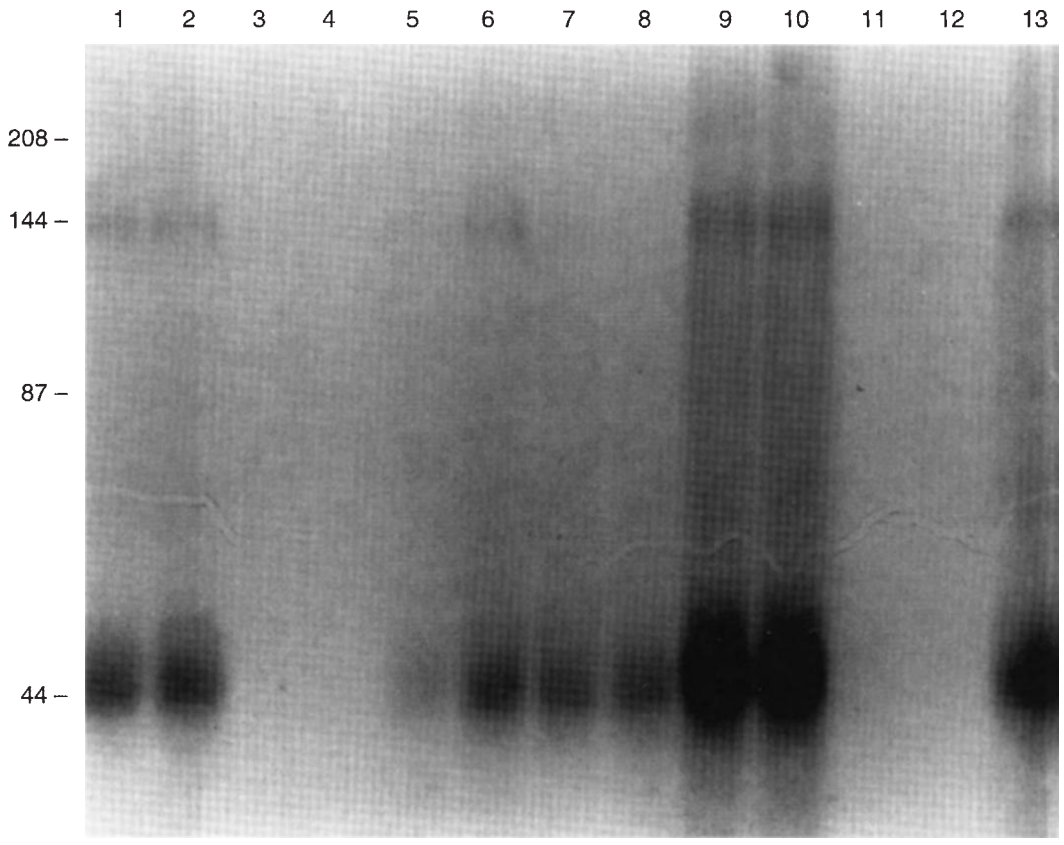

Fig. 4. Autoradiograph of [^{125}I]-IGF-I cross-linked to IGF-I receptors on confluent, quiescent, human proximal tubule cells (PTC) incubated for 24 hours in basic media (control, lanes 1 to 8) or cortical fibroblast (CF)-conditioned media (CF-CM, lanes 9 to 13). Competition with unlabeled IGF-I (1 μM, lanes 3, 4, 11, 12), unlabeled insulin (1 μM, lanes 5, 6, 13) or αIR (5 μg/ml, lanes 7, 8) is shown. The migration positions of molecular weight standards (kDa) are indicated on the left.

sites on their basolateral or interstitial aspects, respond to the presence of CF-CM with augmented growth and transport of Na^+ and H^+. As these effects are blocked by specific IGF-I receptor immunoneutralization, a role of CF-secreted IGF-I is likely. Moreover, PTC-associated IGFBP-3, which is increased in the presence of CF, serves to further facilitate the stimulatory effects of CF-derived IGF-I on PTC. Since we have previously validated the integrity of this experimental system in reproducing the characteristics of normal human PTC and CF [14], it is likely that a paracrine relationship exists between these cells *in vivo* and may be important in the regulation of tubulointerstitial homeostasis.

Previous evidence has suggested that the IGF-I/IGFBP axis is modified in models of renal hypertrophy associated intimately with renal disease [10, 27, 28]. Our results suggest that IGF-I production by CF results in proximal tubular hypertrophy, increased sodium transport and hypermetabolism, which have been independently implicated in the pathogenesis of experimental renal scarring [29]. Importantly, the role of IGFBPs has previously been unclear, as although cell-association of an IGFBP, possibly IGFBP-3, has been demonstrated *in vivo* in the hypertrophying kidneys of rats with diabetic nephropathy [28], IGFBPs have been variously reported to either potentiate [30, 31] or inhibit [31] the actions of IGF-I, depending on species and experimental conditions. Our results clearly confirm that the actions of IGF-I were facilitated by human PTC-associated IGFBPs, and establish their importance in the regulation of the human tubulointerstitial growth response.

Prior studies have suggested that glomerular mesangial and cortical collecting duct cells are the primary renal sites of production of IGF-I [11, 32]. However, with the measured rate of IGF-I synthesis by CF in the present study (42.6 ± 4.95 ng/10^6 cells/day) being similar to that reported for cortical collecting duct cells (48.9 ± 52 ng/10^6 cells) [33] and much higher than observed production rates by mesangial cells (~ 6 ng/10^6 cells/day) [32], our finding indicates that IGF-I secretion by CF is physiologically significant. Although IGF-I produced by CF may act in an autocrine fashion, the close proximity of CF to PTC *in vivo* suggests that CF may constitute an important paracrine source of IGF-I for the human proximal tubule.

Our hypothesis that a functionally significant IGF-I paracrine system exists between neighboring CF and PTC is supported by the findings that PTC express IGF-I receptors and that occupancy of these receptors by CF-derived IGF-I induces biological responses with regards to both growth and transport. The affinities and maximum specific binding capacities of apical and basolateral membranes for IGF-I in the present study are comparable with those reported for rat and canine PTC [34, 35], although no previous studies in human tissue are available. As with all other animal PTC studied, human PTC exhibit an asymmetrical distribution of IGF-I receptors, which are predominantly located on

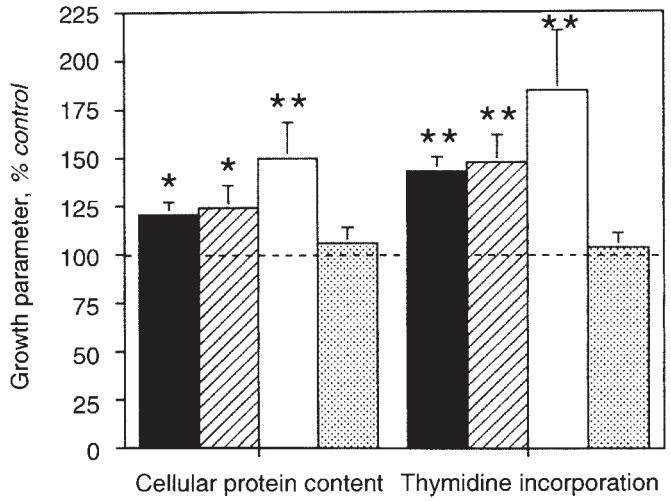

Fig. 5. Cellular protein contents and thymidine uptakes in confluent proximal tubule cells (PTC) exposed for 24 hours to cortical fibroblasts (CF)-conditioned media (CF-CM, ■, $N = 30$), dialyzed CF-CM (▨; $N = 30$), cocultured CF (□; $N = 24$) or PTC-conditioned media (PTC-CM; ▨; $N = 24$). Results are expressed as a percentage of controls (cells incubated in basic media alone). $^*P < 0.05$ versus control, $^{**}P < 0.01$ versus control.

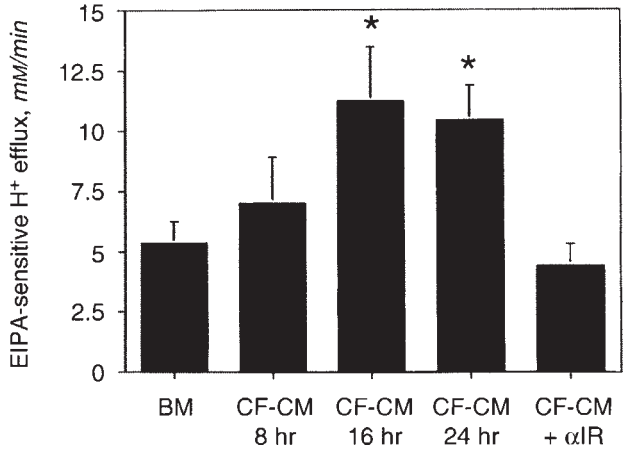

Fig. 6. Time course for stimulation of proximal tubule cell (PTC) sodium-hydrogen exchange (NHE) activity by cortical fibroblasts (CF)-conditioned media (CF-CM) and its inhibition by IGF-I receptor immunoneutralization. Confluent PTC were exposed to basic media (BM); to CF-CM for 8, 16 or 24 hours (CF-CM 8 hr, CF-CM 16 hr and CF-CM 24 hr, respectively); or to CF-CM containing 5 μg/ml αIR for 24 hours (CF-CM+αIR). NHE activity was measured as the component of H^+ efflux following cellular acidification which was inhibited by 10 μM EIPA. $^*P < 0.05$ versus BM and CF-CM+αIR.

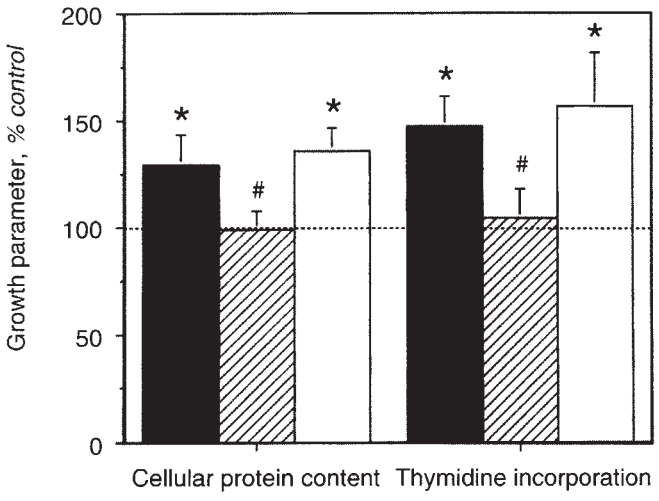

Fig. 7. Effect of IGF-I receptor immunoneutralization on growth effects of cortical fibroblasts (CF)-conditioned media (CF-CM) on proximal tubule cells (PTC). Confluent, quiescent PTC were incubated for 24 hours in CF-CM alone (CF-CM; ■; $N = 30$), in CF-CM plus 5 μg/ml αIR (CF-CM+αIR, ▨; $N = 30$), or in CF-CM plus 5 μg/ml mouse non-immune globulin (CF-CM+MNIG, □; $N = 30$). Cellular protein content and thymidine incorporation were then measured. Results are expressed as percentages of controls (basic media alone). $^*P < 0.05$ versus control, $\#P < 0.05$ versus CF-CM.

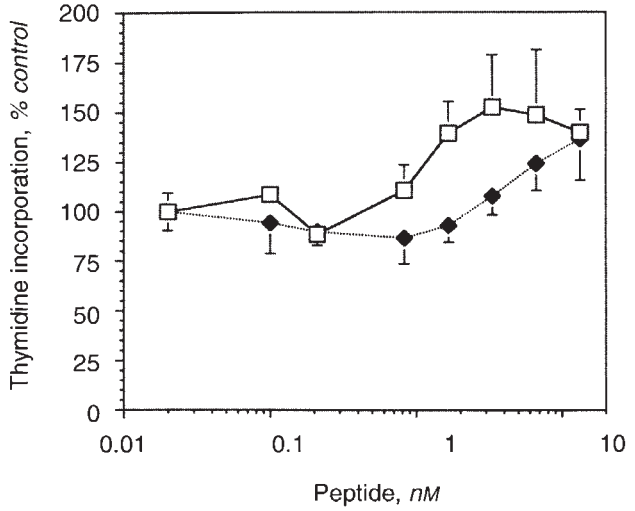

Fig. 8. Comparison of effects of IGF-I (□) and des(1-3)IGF-I (◆) on proximal tubule cell (PTC) DNA synthesis. Confluent, quiescent PTC were incubated for 24 hours in serum-free media containing various concentrations of IGF-I or des [1-3]IGF-I. Results are normalized against values for controls (vehicle alone) and represent the mean ± SEM of 6 experiments in triplicate.

basolateral membranes. Since these basolateral membranes lie in close contact with the renal cortical interstitium *in vivo*, the predominance of IGF-I receptors in these regions may underline the importance of CF as a source of endogenous IGF-I.

Exposure of PTC to CF-CM additionally results in stimulation of PTC growth and NHE activity, a finding that is reproduced by the administration of exogenous IGF-I [7]. The stimulation of PTC growth is even greater when PTC are cocultured with CF. This augmentation may reflect a difference in PTC response to the sustained release of IGF-I by CF in coculture, as opposed to the presentation of a single bolus of endogenous IGF-I in CF-CM. Alternatively, a positive feedback loop may exist between CF and PTC in coculture. The key role of IGF-I in mediating the stimulation of PTC DNA synthesis, cellular hypertrophy and NHE activity by CF-CM is confirmed by the fact that co-incubation with blocking anti-IGF-I receptor antibody abrogates these effects. This is in keeping with *in vivo* studies, which have found that experimentally induced renal growth is preceded by

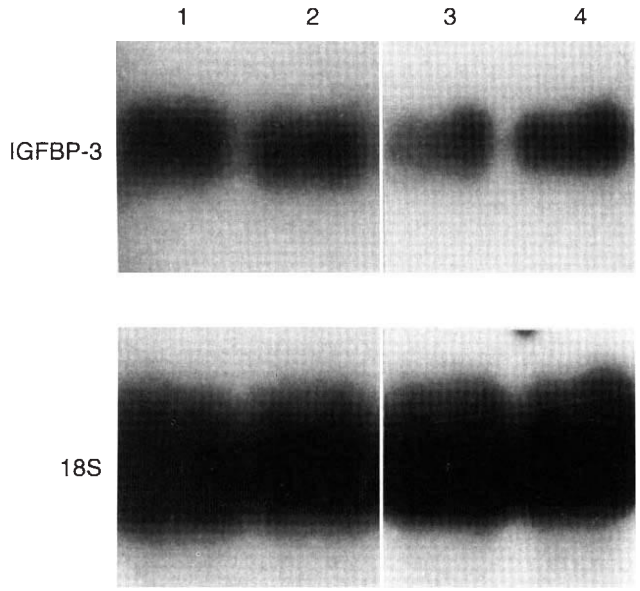

Fig. 9. Northern blot analysis of IGFBP-3 mRNA in proximal tubule cells (PTC; lanes 1 and 2) and cortical fibroblasts (CF; lanes 3 and 4). PTC were incubated for 24 hours in basic media (lane 1) or CF-CM (lane 2). CF were incubated for 24 hours in basic media (lane 3) or PTC-CM (lane 4). Extracted RNA was resolved on an agarose/formaldehyde gel, transferred to a nylon membrane and hybridized to random-primed ^{32}P-labeled cDNAs for IGFBP-3 and 18S.

increases in both NHE activity and in whole kidney IGF-I mRNA and protein levels [9, 36].

Although IGFBPs have been clearly recognized as having both inhibitory and stimulatory effects on IGF-I action in different experimental settings [30, 31], their role in determining the action of IGF-I in tubulointerstitial renal disease has not been studied. A previous study in rats indicated that CF solely express IGFBP-3 mRNA and that PTC exclusively express IGFBP-4 mRNA [37]. However, the present investigation found that human CF produce and secrete large quantities of both IGFBP-3 and IGFBP-2, while human PTC synthesize IGFBP-3. Interestingly, the fate of IGFBP-3 differs between the two human cell populations in that IGFBP-3 produced by CF is largely secreted, while PTC IGFBP-3 remains principally cell-associated and is not released into PTC-CM in detectable quantities. Moreover, the finding that des(1-3)IGF-I, an IGF-I analog that has a comparable receptor affinity but markedly decreased affinity for IGFBPs [8], is significantly less potent than IGF-I in stimulating PTC growth suggests that cell-associated IGFBP-3 facilitates IGF-I action. One possible mechanism for this potentiation of IGF-I activity may involve a lowering of the affinity of PTC-associated IGFBP-3 for IGF-I, thereby promoting its release in the vicinity of the PTC IGF-I receptor. The association constant (K_a) of cell-associated IGFBP-3 for IGF-I has previously been shown in bovine fibroblasts to be tenfold lower than that of soluble IGFBP-3 and is nearly equivalent to that of the IGF-I receptor [30]. This finding is corroborated in human PTC by the observation of linear Scatchard plots, despite the demonstration by affinity labeling experiments of two IGF-I-binding sites (that is, cell-associated IGFBP-3 and the IGF-I receptor).

The release of two different types of IGFBP by CF may also be important in determining the actions of IGF-I on PTC. This release is differentially regulated since exposure of CF to PTC-CM resulted in an increase in IGFBP-3 secretion without alteration in IGFBP-2 or IGF-I levels. The implications of modulation of tubulointerstitial IGFBP subtypes for local IGF-I bioactivity remain to be determined. However, the relatively greater effects of CF on PTC in coculture compared with conditioned media experiments may partly relate to augmented IGF-I action on PTC via stimulated CF secretion of IGFBP-3. Since exposure of PTC to CF-CM was not associated with an increase in PTC IGFBP-3 mRNA, the increase in PTC-associated IGFBP-3 may represent increased binding of CF-secreted IGFBP-3 to PTC surfaces.

An advantage of the present experimental protocol is that it is performed on human cells, which have been shown to reproduce the *in vivo* characteristics of human PTC and CF. Prior *in situ* hybridization studies in rat kidney have failed to localize IGF-I mRNA to the cortical interstitium [38]. One possible reason for the apparent absence of IGF-I production in rat CF, while significant amounts are manufactured by human cells, could be that *in situ* hybridization is a relatively insensitive technique for detecting IGF-I production in a small population of cells, which make up less than 4% of the total kidney volume and are evenly distributed throughout normal renal cortex [2]. Moreover, significant species differences may exist in the IGF-I/IGFBP axis, thereby making extrapolations from animal studies to humans potentially unreliable. Indeed, cellular patterns of IGF-I and IGF-II gene expression have been reported to differ between rat and human kidneys [39].

Although CF modulation of PTC growth and function occurred largely via the IGF-I/IGFBP axis *in vitro*, tubulointerstitial "cross-talk" is likely to be more complex *in vivo* with additional influences being exerted by an interplay of ambient growth factors [23]. For example, IGF-I and IGFBP production have been shown to be influenced by a variety of cytokines known to be important in the response to renal injury, including transforming growth factor-β (TGFβ) [40], angiotensin II [41] and epidermal growth factor (EGF) [42]. IGF-I in turn has been shown to control the actions of other growth factors, such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), angiotensin II and EGF [43–45].

In conclusion, we have demonstrated that there is a specific paracrine interaction between CF and PTC, which involves the IGF-I/IGFBP axis. The production of IGF-I by human CF is likely to play a key role in the regulation of human proximal tubule growth and sodium transport. The concomitant release of IGFBP-3 by CF is subject to reciprocal regulation by PTC and is associated with an increase in PTC-associated IGFBP-3, which in turn facilitates the actions of IGF-I. Our studies suggest that the renal tubulointerstitial IGF-I/IGFBP axis may be an important pathogenetic factor in the deranged tubulointerstitial growth found in progressive renal diseases.

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APPENDIX

Abbreviations used in this article are: IGF-I, insulin-like growth factor-I; PTC, proximal tubule cells; CF, cortical fibroblasts; CF-CM, CF conditioned media; NHE, sodium-hydrogen exchange; PTC-CM, proximal tubule cells conditioned media; IGFBP, insulin-like growth factor binding proteins.

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